APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1989, p. 2101–2104
0099-2240/89/082101-04$02.00/0

Vol. 55, No. 8

Cadmium Resistance Screening in Nitrilotriacetate-Buffered Minimal Media

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Received 22 February 1989/Accepted 23 May 1989

Media used to determine the MICs of heavy metals for bacteria are unreliable because organic components in the media bind or chelate most of the metal being studied. To define specific metal activity in media and to maintain metal activity at a constant level, HEPES-MES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid–2-(*N*-morpholine)ethanesulfonic acid] salts medium with arabinose medium was modified, and the modified medium was used to examine the MIC of cadmium for *Rhizobium fredii* USDA 201. Arabinose-HEPES-MES was modified by addition of the chelator nitrilotriacetate to buffer the supply of free Cd^{2+} ion to maintain a constant Cd activity and by the use of only MES to buffer pH (buffered arabinose-MES medium [BAM]). Ca and Mg were supplied at the normal levels for soil solutions, and other trace elements were supplied at the levels required for normal growth of plants. The concentration of free Cd^{2+} ion was calculated by using the computer program GEOCHEM-PC with a corrected data base. The Cd MIC in BAM was 14.0 μM, while that in a tryptone-yeast extract medium was 107 μM. The results indicate that substantial free Cd^{2+} is removed from solution in most standard media, resulting in falsely high MICs. The new BAM medium allows for the precise determination of MICs, thus avoiding the uncertainties associated with other media.

Numerous reported toxicological studies have examined the heavy-metal sensitivity or resistance of bacteria isolated from a number of habitats. The heavy-metal MICs for bacterial isolates from soil (10, 20), leaf litter (18), water (1), and sewage sludge-amended soil (17) have all been studied. To determine the MICs of heavy metals, most studies have utilized the medium which best supports the growth of the specific organism or group or organisms. Media were amended with varied amounts of heavy-metal salts and inoculated with the appropriate organisms, and growth was measured to determine the MIC.

A number of problems are associated with this approach. The most important limitation is that one never knows the actual metal activity in the test medium. The activity of the free metal ion, ordinarily considered to be the toxic metal species which ultimately determines the microbial response to the metal, rarely approaches the total metal concentration added to the medium. Microbial uptake, chelation to various components of the medium, ion complexes, and sorption on the cells can each cause a reduction in free metal activity.

Of particular importance is the binding or chelation of metals to unspecified organic components found in most biological media. For example, Babich and Stotzky (2) used nutrient agar (Difco Laboratories) containing 5 g of peptone and 3 g of beef extract per liter when studying the Cd sensitivity of soil bacteria. Borges and Wollum (5) utilized a mineral salts medium containing 0.25 g of yeast extract per liter to examine the Cd sensitivity of *Bradyrhizobium japonicum*. Organic compounds in these uncharacterized medium components could bind significant quantities of the Cd added to the medium.

Binding of metal salts to the undefined organic constituents of most biological media was recognized by Kinkle et al. (17). These authors used a complete mineral salts medium without the addition of undefined constituents. However, microbial uptake or sorption of metals, and ion pairs or complexes, prevented their determining the metal activity even in this highly defined medium. Alterations in medium pH around individual colonies on a plate should also affect metal activity. Knowledge of metal activity would be especially important when comparing metal tolerances of bacteria with different growth rates or pH reactions.

From this discussion, it is apparent that MICs determined with traditional media cannot be related to actual metal concentrations in the habitat from which the bacteria were isolated. Further, the differential response of bacteria with respect to growth and acid-base balance make relative comparisons difficult to interpret with currently used media.

Recognizing the problems associated with past approaches to determine heavy-metal effects on microorganisms, our objective was to develop a medium in which the free metal ion concentration could be controlled at known constant levels during growth of the bacteria.

Research has shown that the free ion of a heavy metal (e.g., Cd^{2+}) is the toxic or essential ion. As early as 1957, DeKock and Mitchell (9) found that chelated heavy metals were not toxic to higher plants. Subsequently, Halvorson and Lindsay (14) showed that chelated Zn was not available to plants. Checkai et al. (7) reported that increasing the concentration of total Cd by increasing CdEDTA (Cd chelate with EDTA) caused no change in plant Cd when Cd^{2+} activity was buffered by a resin system, a result similar to that obtained by Sunda et al. with shrimp (22).

Recent advances in computer modeling have made it possible to accurately calculate the free metal ion concentration in solutions. The data base used by the computer program GEOCHEM-PC (21) has been modified by Chaney et al. (6; R. L. Chaney, P. F. Bell, and B. A. Coulombe, Hort. Sci., in press) by the addition of correct formation constants for chelating agents often used in plant nutrition research. Thus, the free metal ion concentration and activity can be calculated and buffered for any defined medium in which formation constants for and total concentrations of all constituents are known.

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We utilized the HM medium of Cole and Elkan (8) as the basis for the formulation of our modified medium. This medium contains (in micromolar) Na_2HPO_4, 880; Na_2SO_4, 1,790; NH_4Cl, 5,980; $MgSO_4 \cdot 7H_2O$, 730; $FeCl_3$, 14.8; $CaCl_2 \cdot 2H_2O$, 88.4; *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (Sigma Chemical Co.), 5,640; and 2-(*N*-morpholino)ethanesulfonic acid (MES) (Sigma), 5,460. The medium pH was adjusted to 6.6 with dilute NaOH. Arabinose was added at 0.1% (AHM).

Medium components were subsequently adjusted to concentrations that we believed to be more appropriate for media which simulate the soil solution in the rhizosphere. Ca, Mg, and other nutrients were added at concentrations normally found in soil solutions. P was added at 10 times typical soil solution levels, but this P did not complex significant amounts of Cd^{2+} at the pH used. The new medium used chelating agents to buffer the supply of microelements and is called buffered arabinose-MES medium (BAM). As prepared, the medium contained (in micromolar) Na_2HPO_4, 100; NH_4Cl, 1,000; $MgSO_4 \cdot 7H_2O$, 1,000; $CaSO_4$, 1,500; $CaCl_2 \cdot 2H_2O$, 1,000; and MES, 10,000. As before, arabinose was added at 0.1%, and the pH was adjusted to 6.6. All components were added to 1.5% molten purified agar in distilled deionized water.

Because we were adding a strong metal chelator, nitrilotriacetate (NTA), to the medium, the chelator could be expected to bind contaminating micronutrients in the solution. This potentially could restrict microbial growth. Therefore, we added micronutrients required to grow plants with normal foliar composition at the following final concentrations (in micromolar): FeEDDHA [Fe chelate with ethylenediamine-di(2-*o*-hydroxyphenylacetate)], 20; $CuCl_2 \cdot 2H_2O$, 2.5; $ZnCl_2$, 2.5; $MnSO_4 \cdot H_2O$, 2.0; H_3BO_3, 10; MoO_3, 0.2; $NiCl_2 \cdot 6H_2O$, 0.5; and $CoCl_2 \cdot 6H_2O$, 0.5. NTA chelates the Cu, Zn, Mn, Ni, and Co added as salts such that, at pH 6.6 pCu = 10.8, pZn = 8.6, pMn = 6.0, pNi = 10.1, and pCo = 9.0. However, because NTA would not keep Fe completely chelated and soluble at pH 6.6, we used a combination of the chelators EDDHA and NTA. EDDHA has a strong selectivity for Fe^{3+}, and added Cd does not displace Fe from FeEDDHA (6; Chaney et al., in press). However, the low strength of chelation of Cd by NTA allowed the buffered Cd concentrations to be physiologically significant. Other chelators were evaluated but bound Cd too strongly for study of Cd toxicity to microbes. A comparison of the free Cd^{2+} concentrations in BAM medium containing 100 μM chelator in excess of the chelated microelements, including the added Cd, is shown in Table 1 for a number of chelators. Compounds such as diethylenetriaminepentaacetate, EDTA, and *N*-2-hydroxyethylethylenediamine-*N*,N^1,N^1-triacetate bound Cd so strongly that toxic levels could not be attained. Some other ligands bound Cd so weakly that buffering could not be attained. NTA provided buffering at levels of free Cd^{2+} which are potentially toxic to bacteria. Most higher plants are severely injured by as little as 1 μM free Cd^{2+}. For reference, soil solutions seldom reach 0.1 μM free Cd^{2+} even after large amounts of sludge-Cd are applied (12). Other chelators, such as iminodiacetate or amino acids, would be metabolized so rapidly that it would be difficult to maintain the free chelator concentration required to buffer Cd and other microelements.

When one uses chelators to buffer free metal ion concentrations, the chelator must be added in excess of the sum of the concentrations of the strongly chelated microelements. In essence, the remaining chelator is filled with Ca, and the competition between solution Ca and microelements for the chelator controls the actual levels of free metal ion obtained.

TABLE 1. Buffering of Cd by different chelators[a]

Ligand	$pCd2^+$ (mol/liter)
Total Cd	4.00
No chelator added	4.53
IDA	4.53
HEIDA	5.09
EDDHA	5.75
NTA	6.08
EDDA	6.20
HBED	7.44
HEDTA	7.62
EGTA	8.35
EDTA	8.53
CDTA	9.43
DTPA	10.57

[a] Cd (100 μM) was added to complete medium with no chelator for microelements and Cd, or CdNTA (100 μM) was added to complete medium with 100 μM chelator in excess of the sum of the microelement concentrations. Fe was added as 20 μM FeEDDHA to all media. Results were for pH 6.6 as calculated with GEOCHEM-PC. Abbreviations: IDA, iminodiacetate; HEIDA, 2-hydroxyethyliminodiacetate; EDDA, ethylenediamine-*N*,*N'*-diacetate; HBED, *N*,*N'*-bis(2-hydroxybenzyl)ethylenediamine-*N*,*N'*-diacetate; HEDTA, *N*-2-hydroxyethylethylenediamine-*N*,*N'*,*N'*-triacetate; EGTA, ethylene-bis(oxyethylenenitrilo)tetraacetate; CDTA, *trans*-1-2,-cyclohexanediaminetetraacetate; DTPA, diethylenetriaminepentaacetate.

Thus, 8 μM NTA was added to bind the Ni, Co, Cu, Mn, and Zn, with the addition of 100 μM excess NTA. A solution of CdNTA (1:1 molar ratio) was then added to obtain free Cd^{2+} ion concentrations of 0, 0.01, 1.00, 1.15, 1.70, 3.16, 5.00, 8.00, 10.0, 12.0, 14.0, and 17.8 μM. The 108 μM NTA and CdNTA solutions were adjusted to pH 6.6, filter sterilized, and added to molten agar.

After preparation of all plates, they were streaked with *Rhizobium fredii* USDA 201. *R. fredii* was initially grown in standard AHM broth to 10^8 cells per ml. Cells were then centrifuged, washed three times in a phosphate buffer, and resuspended in the same buffer. The inoculum was then streaked onto the CdNTA-amended plates. For comparisons, *R. fredii* was also streaked onto yeast extract-mannitol agar (YEM) (23), tryptone-yeast extract agar (TY) (3), and the original AHM containing various concentrations of total Cd salt in the traditional method of determining Cd MICs.

After 6 days of growth, cells were scraped from the plates and washed in basal HM salts plus 108 μM NTA for 2 min to remove contaminating exocellular Cd. The cells were then freeze-dried and weighed. Cells were digested in a nitric acid-perchloric acid mixture as described by Isaac and Kerber (16). The cellular Cd concentration was then measured with a Perkin-Elmer 5000 atomic absorption spectrophotometer. All experiments were repeated three times.

The Cd MIC results as determined on BAM, AHM, TY, and YEM are presented in Table 2. The Cd MICs for *R. fredii* USDA 201 on BAM, AHM, TY, and YEM were 14.0, 53.4, 106.8, and 71.2 μM, respectively. The Cd MICs determined in each of the three replicate experiments were identical. Differences in the Cd MICs can be related to the medium pH as well as to the quantity of undefined nutritional components added to the medium. AHM has a pH of 6.6 and no undefined nutritional components except agar. Agar is a galactan sulfate polymer; the sulfate is nonselective in binding Ca and Cd, so agar does not interfere with Cd^{2+} toxicity if a medium contains the levels of Ca found in soil solutions of most soils. Some free Cd^{2+} ion would be removed from solution by ion complexing (with Cl, SO_4, etc.) and by microbial uptake or adsorption on cells. For YEM, the

TABLE 2. Growth and cellular Cd concentrations of *R. fredii* USDA 201 on four media supplemented with various amounts of Cd

Cd in medium (μM)	Cellular Cd (μg/g [dry wt]) in[a]:			
	BAM	AHM	TY	YEM
0.0[b]	<1.0	<1.0	1.05	1.07
0.01[b]	34.0	ND	ND	ND
1.00[b]	152.0	ND	ND	ND
1.78[b]	156.0	ND	ND	ND
3.16[b]	154.0	ND	ND	ND
5.00[b]	291.0	ND	ND	ND
8.00[b]	792.0	ND	ND	ND
10.0[b]	812.0	ND	ND	ND
12.0[b]	+	ND	ND	ND
14.0[b]	−	ND	ND	ND
17.8[c]	−	335.0	197.0	171.0
35.6[c]	ND	+	493.0	+
53.4[c]	ND	−	461.0	+
71.2[c]	ND	−	691.0	−
89.0[c]	ND	−	+	−
106.8[c]	ND	−	−	−

[a] ND, Not determined; +, growth noted but insufficient to determine cellular content; −, no growth.
[b] Buffered Cd^{2+} added as CdNTA with 100 μM free NTA.
[c] $CdCl_2$ added to the medium. Free Cd^{2+} activity unknown.

medium pH is 6.6 and 1.0 g of yeast extract per liter was added to the medium. The addition of yeast extract would be expected to chelate a portion of the Cd and thus result in a higher MIC. The highest MIC was observed with the TY medium. This medium has a pH of 7.3 and contains 5.0 g of yeast extract plus 3.0 g of tryptone per liter. These additions would be expected to chelate significant quantities of Cd, and the higher pH would also favor Cd chelation. Thus, Cd MICs were higher in all other media than in the BAM medium, most likely because of chelation, complexing, and cellular uptake and sorption, which reduce the free Cd^{2+} ion concentration.

While YEM and TY are the standard media which have been used in the past to test heavy-metal effects on rhizobia, most authors have recognized that metal chelation is a significant source of error. Recently, El-Zamik and Wright (11) suggested that yeast extract chelates Fe and that the addition of chelated Fe (200 μM FeEDTA) was required. FeEDTA is a weak chelator of Fe. At a pH above 6, GEOCHEM predicts that Cd quantitatively displaces Fe from FeEDTA, causing $Fe(OH)_3$ to precipitate in the medium. Much lower levels of Fe chelates have been used successfully to grow many microbes; the 20 μM FeEDDHA used in BAM keeps Fe soluble and supports rapid growth of *R. fredii*.

The cellular Cd concentration as determined in cells grown on BAM remained relatively constant up to a Cd^{2+} concentration of 3.16 μM. Beyond this concentration, the cellular Cd level rapidly increased. The highest cellular Cd level observed was 812 μg/g. When the medium Cd^{2+} concentration exceeded 3.16 μM, it is likely that the protective mechanisms of the cells were initiated. Two primary mechanisms by which cells protect themselves from the toxic effects of Cd have been proposed. Exclusion, as mediated by extracellular polysaccharide slime, has been shown to allow *Klebsiella aerogenes* to grow on media containing high levels of Cd (4). Exclusion has also been reported for *Escherichia coli* (19). This mechanism could protect the cell from excessive uptake of Cd until a threshold level is exceeded. The Cd concentrations in cells grown at 8.00 and 12.0 μM Cd^{2+} were very high, indicating significant uptake. At these medium Cd^{2+} concentrations, it is possible that Cd-induced, low-molecular-weight, cysteine-rich Cd-binding peptides are produced to protect the cell. The production of these proteins, called phytochelatins, has recently been reported for *Pseudomonas putida* (15) and *Schizosaccharomyces pombe* (13).

In the current study we modified an existing medium for the determination of the effects of Cd and other heavy metals on bacteria. The novel features of the new medium include the use of GEOCHEM-PC to predict the free metal ion concentrations in the medium and the use of a chelated supply of Cd to buffer and maintain the Cd^{2+} concentration during an experiment. Given that all media previously used for the determination of heavy-metal effects on bacteria have significant limitations, this new BAM methodology represents an important improvement in the study of heavy-metal–bacterial interactions. Although we tested only Cd in these experiments, chelate-buffered nutrient solutions have been used to control free Zn, Cu, Mn, Ni, and Co ion levels in the same manner as used here for Cd. Thus, the BAM medium should be applicable to all metals except Pb, with which P levels in the medium would cause precipitation.

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